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(57) Abstract

The invention generally relates to methods of enhancing the growth of pearl producing mollusks through the exposure of the mollusk to a growth enhancing agent. Also provided are transgenic mollusks capable of expression of exogenous growth enhancing agents, and methods of culturing pearls comprising the use of these methods and mollusks.

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ENHANCING GROWTH AND PEARL PRODUCTION IN MOLLUSKS

The present invention generally relates to methods of enhancing the growth of pearl-producing mollusks through the exposure of the mollusks to growth enhancing agents. Also provided are transgenic mollusks capable of expression of exogenous growth enhancing agents, and methods of culturing pearls.

BACKGROUND OF THE INVENTION

The pearl has long been considered a prized jewel valued for both its lustre and brilliance. Despite its customary classification as a "gem," the pearl is a product of animal origin, being produced by mollusks. A pearl is generally formed when a foreign body becomes embedded in the tissue of a mollusk. Unable to dislodge the foreign body, the mollusk coats it with a calcium carbonate composition which is secreted from its epithelial, or "mantle" tissues. *J. Taburiaux, Pearls: Their Origin Treatment and Identification*, (D. Ceriog-Hughes, Trans., Chilton Book Co., 1986). This secreted material is termed the "nacre." After a prolonged period, the foreign body is encased in the nacre and has become what is commonly known as a pearl.

Because the production of "natural pearls" relies entirely upon the happenstance occurrence of the embedding of a foreign body into the epithelial tissue of an oyster, yields of such natural pearls on a per oyster basis are low. In response to a higher worldwide demand for pearls, oyster farmers began artificially introducing foreign bodies into the tissue of the oyster. Pearls resulting from this process are termed "cultured pearls." By culturing pearls, farmers could ensure that the oyster possessed all of the starting materials necessary to create a pearl. Furthermore, by utilizing a foreign body of a desired size and shape, farmers could, to

some extent, control the final product of the pearl culturing process. See, *Taburiaux*, supra.

Modern commercial pearl culturing is practiced using several species of pearl oysters and other mollusks, including abalone, clams and mussels. The pearl oysters are collected from the wild as adults, caught in the wild as juveniles, or "spat", in spat collectors, or can be produced in hatcheries by spawning broodstocks. The oysters are then raised for use in the production of pearls. Generally, spat or juvenile oysters, also described as pre-adult oysters, require culturing of at least one year before they reach adult size and are large enough to be used for pearl production.

In the typical pearl culturing process, a foreign body, or "nucleus," is implanted into the gonadal tissue of a mollusk, typically an oyster species. Where "mabe" pearls are being produced, this nucleus is attached directly to the shell of the mollusk, directly contacting the mantle tissue of the acceptor mollusk. The mantle is a fold of epithelial tissue which surrounds the collection of organs and tissue between the shells, or valves, of the oyster. These organs and the mantle are herein referred to collectively as the "body" of the mollusk. The outer portion of this mantle is generally responsible for periostracum formation and nacre production.

In culturing traditional pearls, a small piece of mantle tissue from a separate, or "donor" mollusk is implanted in the mollusk concurrently with the implantation of the nucleus. The mollusk into which the nucleus and, optionally, the donor tissue is implanted is generally termed the "acceptor" shell or mollusk. Without being bound to any theory, it is believed that the donor mantle tissue surrounds the nucleus as the "pearl sac" and secretes the nacre which forms the pearl, while the acceptor mollusk acts as a nutritive repository for the process. See, *Taburiaux*, supra.

The time from implantation of the nucleus to harvesting of the pearls can take from eighteen months to three years. Aside from the costs associated with this lengthy process, the large amount of time required for pearl culturing also increases the risk that an oyster will not survive to complete

the process or that a flaw will be created in the forming pearl, such as the incorporation of off-colored matter, an alteration of pressure on the forming pearl or an aggregation of two forming pearls resulting in a change in the pearls' shape. This risk is further increased where larger pearls are desired. Such larger pearls naturally require larger mollusks and a longer culture time to form. Finally, because the price of pearl oysters is high, it is desirable to "recycle" these oysters for multiple pearl cultures. However, currently, only approximately one third of pearl oysters are recultured. See, *Taburiaux*, supra. It is thus desirable to reduce the time requirements in spat production and culture, and in the pearl culturing processes.

Exposure of the eastern oyster, *Crassostrea virginica*, to a class of growth enhancing bioactive peptides has been reported to increase the growth rates of both juvenile and adult oysters. Paynter & Chen, *Biol. Bull.* 181: 459-462 (1991). In particular, exogenous treatment of these oysters with rainbow trout growth hormone was reported to increase both shell height and tissue weight over those oysters in the control group.

Additionally, the use of growth regulating hormones, including those of mammalian origin, such as insulin and growth hormone, have been reported to be effective in accelerating the growth rate of post-larval abalone. See, Morse, *Aquaculture*, 39:263-282 (1984).

The present invention provides methods of enhancing the growth rates of pearl-producing mollusks, and also provides mollusks having enhanced growth rates. Increased growth rates lessen the time required for spat to be cultured prior to their use in pearl production. Additionally, an increase in the growth rate of a pearl producing mollusk results in an increase in the rate of nacre production around implanted nuclei, thus reducing the culturing time for the pearl. Such a decrease in culturing time also increases the number of mollusks that can be recultured, further reducing costs. Therefore, the yield of pearl producers is increased through faster preculturing and culturing processes, recycling of

pearl mollusks and reduced flaws in the pearls produced. Further, the potential size of the pearls produced is also be increased.

SUMMARY OF THE INVENTION

One embodiment of the present invention provides a method of enhancing the growth of a pearl producing mollusk. The method comprises introducing into the germline of the mollusk a nucleic acid sequence, which comprises a segment encoding a growth enhancing agent operably linked to a promoter sequence whereby the segment is capable of expressing the growth enhancing agent in the mollusk.

In an alternate embodiment, the present invention also provides a transgenic mollusk wherein the mollusk's germline comprises an exogenous nucleic acid sequence. This exogenous nucleic acid sequence comprises a segment which encodes a growth enhancing agent, and is capable of expressing the growth enhancing agent in the mollusk.

In an additional embodiment, the present invention also provides a method of culturing pearls. This method comprises the steps of introducing a nucleus into an acceptor mollusk. Optionally, the method may comprise the concurrent introduction of mantle tissue from a donor mollusk into the acceptor mollusk. The acceptor mollusk is exposed to an effective amount of a growth enhancing agent and cultured. Following culturing, pearls are harvested from the acceptor mollusk.

In preferred embodiments, the acceptor mollusk is exposed to the growth enhancing agent by immersing the acceptor mollusk in a solution comprising the growth enhancing agent at a concentration of from about 1 nM to about 10 mM of the growth enhancing agent, and more preferably, at a concentration of from about 0.1 μM to about 100 μM of the growth enhancing agent. Such exposure is generally carried out from about monthly to weekly to about daily.

In a related embodiment, the acceptor mollusk is exposed to the growth enhancing agent by inserting a composition comprising the growth enhancing agent into the interior of the

acceptor mollusk. Insertion of the composition comprising the growth enhancing agent is carried out, for example, by injecting the composition into the interior of the mollusk, or by manually placing the composition in the interior of the mollusk. Such compositions optionally comprise the growth enhancing agent free in solution or incorporated in a matrix, such as a controlled release composition whereby a constant low-level release of the growth enhancing agent occurs.

In a further embodiment, the present invention also provides a method of culturing pearls comprising the steps of introducing into an acceptor mollusk, a nucleus and mantle tissue from a donor mollusk, where the donor mollusk comprises an exogenous nucleic acid sequence which encodes a growth enhancing agent, said sequence being operably linked to a promoter sequence whereby said mantle tissue from said donor is capable of expressing said growth enhancing agent. The acceptor mollusk is then cultured. Following this culturing, pearls are harvested from the acceptor mollusk.

Alternatively, in another embodiment, the present invention provides a method of culturing pearls comprising the steps of introducing a nucleus into an acceptor mollusk, and optionally comprising concurrently introducing mantle tissue from a donor mollusk into said acceptor mollusk, said acceptor mollusk comprising an exogenous nucleic acid sequence which encodes a growth enhancing agent, said sequence being operably linked to a promoter sequence whereby the acceptor mollusk is capable of expressing the growth enhancing agent; culturing said acceptor mollusk; and harvesting pearls from said acceptor mollusk.

In still another embodiment, the present invention provides a method for culturing pearls comprising exposing a mollusk to an effective amount of a growth enhancing agent prior to the introduction of a nucleus and optionally the donor mantle tissue. Following introduction of the nucleus, and optionally, the mantle tissue from a donor mollusk, into the interior of the acceptor mollusk, the acceptor mollusk is cultured, and pearls are harvested therefrom. Preferably, at

the time of the exposure, the mollusk is a juvenile, larval or embryonic mollusk.

For the various embodiments of the present invention, the growth enhancing agent is preferably selected from the group consisting of insulins, insulin-like growth factors and growth hormones. More preferably, the growth enhancing agent is porcine, bovine or human growth hormone or insulin. Most preferably, the growth enhancing agent is porcine, bovine or human insulin.

For those embodiments comprising an exogenous nucleic acid sequence operably linked to promoter sequences, preferred promoter sequences are selected from the group consisting of RSV-LTR, actin and metallothionein promoter sequences.

The preferred mollusks of the embodiments of the present invention are selected from the group consisting of *Pinctada maxima, Pinctada margeritifera, Pinctada martensi fucata, Pinctada radiata, Pinctada vulgaris, Pinctada fucata, Pinctada maculata, Pinctada albina*, *Pteria penguin*, *Unionides* sp. *and Haliotis* sp. For those embodiments comprising an acceptor mollusk and a donor mollusk, such mollusks are selected independently from this group.

DESCRIPTION OF THE PREFERRED EMBODIMENT

I. <u>General</u>

An object of the present invention is to provide methods of enhancing the growth of pearl producing mollusks as well as provide mollusks having such enhanced growth characteristics. These methods and mollusks are useful in the pearl culturing process to reduce processing time and increase size and number yields of pearl producing mollusks.

Mollusks which are particularly useful in the present invention include oyster species such as *Pteria penguin, Pinctada maxima, Pinctada margeritifera, Pinctada martensi fucata, Pinctada radiata, Pinctada vulgaris, Pinctada fucata, Pinctada albina and Pinctada maculata*. Other pearl-producing mollusks including abalone, from the *Haliotis* genus and fresh water mussels of the *Unionides* genus. *See Taburiaux*, supra. The term mollusk is used generically to include adult

mollusks, as well as juvenile, larval and embryonic forms thereof.

Mollusks having enhanced growth characteristics generally show an increase in one or more of the following characteristics compared with mollusks having normal growth characteristics: shell height, shell length, overall weight or tissue mass. An enhanced growth rate for a mollusk is the change in mass, length or height per unit time of about 1-150, 10-100 or 20-50% greater than that of a normal mollusk of similar size and age in a similar environment. For example, a normal oyster exhibiting an increase in shell length of 10mm/month, would have that rate of increase enhanced to from about 10.1 mm/month to about 25 mm/month.

Further, mollusks whose growth rate is enhanced also have increased periostracum formation and shell deposition over those mollusks without an enhanced growth rate. Such increased periostracum formation and shell deposition also increase the rate of nacre production which results in an increase in the rate of pearl formation. Additionally, mollusks having an enhanced growth rate, having achieved a larger size, are capable of harboring larger nuclei and/or pearls within their interior. Thus, pearls produced by a mollusk whose growth rate is enhanced or within tissue capable of expressing a growth enhancing agent according to the methods of the present invention generally have a diameter of from about 5 % to about 50 % greater than pearls formed by a mollusk whose growth rate is not enhanced, when cultured under similar conditions for a similar time period. Thus, where a pearl cultured in a nonenhanced growth mollusk is about 10 mm in diameter, a pearl cultured in a mollusk having an enhanced growth rate over the same period is from about 10.5 to about 15 mm in diameter. Alternatively, the mollusk having the enhanced growth rate produces a pearl of given diameter in diameter in about 5-30% less time than a mollusk having a normal growth rate.

Growth enhancing agents generally include those compounds which when exposed to living organisms, directly or indirectly induce the growth of the organism, thereby increasing the

organism's growth rate over and above the natural growth rate of that organism. Thus, compounds essential to the existence of the organism, i.e. nutrients, oxygen or water, are not included within the definition of growth enhancing agents. Examples of growth enhancing agents include growth hormones, growth factors, insulins and insulin-like peptides. Additionally, growth enhancing agents include those factors which induce the release of natural growth inducers within the mollusk. These endocrinological "triggers" result in increased expression of the mollusk's natural growth inducers, thus resulting in an enhanced growth rate. These triggers of growth related hormones have been described in other vertebrates.

Specific growth hormones which are useful in the present invention include fish growth hormones, i.e., rainbow trout (Agellon and Chen, *DNA* 5:463-471 (1986)), coho salmon (Villasenor, et al, *Gene* 65:239-246), and catfish (Tang, et al., *Mol. Mar. Biol. and Biotech.*, 2(4):198-206), as well as bovine, porcine and human growth hormones. Similarly, insulins which are particularly useful in the present invention include mammalian insulins, such as porcine, bovine and human insulin. See, e.g., Plisetskaya, et al., *Gen. Comp. Endocrin.*, 35:133-145 (1978), Morse, D.E., *Aquaculture*, 39:263-282 (1984). Insulin-like peptides, such as human and molluscan insulin-like peptides ("MIP"), are also useful as growth enhancing agents in the methods and mollusks of the present invention. See, e.g., Smit, et al., *J. Mol. Endocrin.*, 11:103-113 (1993), Smit, et al., *Mol. Brain Research*, 14:7-12 (1992). Also included are analogs or amino acid sequence variants of these agents.

II. Method for Enhancing Mollusk Growth by Transgenic Expression of an Exogenous Growth Enhancing Agent and Mollusks Capable of Such Expression

The invention provides methods for enhancing the growth rate of a mollusk and a mollusk having an enhanced growth rate. The mollusks are produced and the methods are practiced by introducing into the mollusk an exogenous nucleic acid

sequence which comprises a segment which encodes a growth enhancing agent operably linked to a promoter sequence, whereby the mollusk is capable of expressing the growth enhancing agent.

A DNA segment is operably linked when placed into a functional relationship with another DNA segment. For example, a promoter or enhancer is operably linked to a coding sequence if it stimulates the transcription of the sequence; DNA for a signal sequence is operably linked to DNA encoding a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide. Generally, DNA sequences that are operably linked are contiguous, and in the case of a signal sequence both contiguous and in reading phase. However, enhancers need not be contiguous with the coding sequences whose transcription they control. Linking is accomplished by ligation at convenient restriction sites or at adapters or linkers inserted in lieu thereof.

An exogenous DNA segment is one foreign to the cell, or homologous to the cell, but in a position within the host cell genome in which the element is not ordinarily found. For example, exogenous DNA segments include heterologous nucleic acid sequences derived from species, genera, families and the like, other than the organism into which it is introduced. Exogenous DNA segments also include a homologous coding sequence operably linked to a heterologous promoter sequence, or alternatively, a heterologous coding sequence operably linked to a homologous promoter sequence. Exogenous DNA segments further include a multiple gene copy nucleic acid sequence where such is not present in the native genome. The presence of multiple copies can result in increased production of the growth enhancing agent relative to expression in the native genome. Exogenous DNA segments are expressed to yield exogenous polypeptides.

In preferred embodiments, the nucleic acid sequence encoding the growth enhancing agent is selected from the group of nucleic acids which encode growth hormones, insulins and insulin-like peptides. The nucleic acid sequences for a variety of growth hormones have been reported. See, e.g.,

Agellon and Chen, DNA 5:463-471 (1986), Villasenor, et al, *Gene* 65:239-246, and Tang, et al., *Mol. Mar. Biol. And Biotech.*, 2(4):198-206. The nucleic acid sequences for insulin related peptides, and specifically for Human and Molluscan Insulin-like Peptide, have also been reported. See, e.g., Smit, et al., *J. Mol. Endocrin.*, 11:103-113 (1993), Smit, et al., *Mol. Brain Research*, 14:7-12 (1992). Similarly, the nucleic acid sequences for various insulins, e.g., human, porcine and bovine, have been reported and are also available from GenBank. Other nucleic acids encoding growth enhancing agents of the present invention can be synthesized, or directly cloned from genomic libraries using, for example, PCR methods. See, e.g., Alberts, et al., *Molecular Biology of the Cell* (2nd Ed. 1989), Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (C.S.H.P. 2d ed. 1989). The nucleic acid sequence encoding the growth enhancing agent can be genomic, cDNA, minigene (i.e., genomic without selected intronic regions not required for expression) or a hybrid of any of these.

Once cloned, the desired sequence is coupled to an appropriate promoter sequence. Promoter sequences useful in the present invention include those of prokaryotic or eucaryotic origin. Generally, promoters that are functional in mammalian cells are also functional in lower eucaryotic forms such as fish or mollusks. Preferred promoter sequences are the long terminal repeat sequence of the Avian Rous Sarcoma Virus (RSV-LTR) (see, e.g., Chen, et al., *Mol. Marine Biol. and Biotech.*, 2(2):88-95 (1993), Dunham, et al., *Mol. Marine Biol. and Biotech.*, 1(4/5):380-389 (1992)), the inducible mouse metallothionein-I promoter, as well as the actin, *pgk*, *tk*, and *dhfr* promoters. Optionally, the coding sequence is operably linked to additional control sequences, such as an enhancer, a 3' and/or 5' untranslated region, a 3' and/or 5' flanking region, an intronic sequence, a signal sequence capable of directing secretion of the growth promoting factor from the cell in which it is expressed, and a polyadenylation site. Additional regulatory sequences are

often obtained from sequences naturally flanking the coding sequence of the growth promoting factor and/or the promoter.

In some transgenes, the sequence encoding the growth enhancing agent is fused in-frame to a second exogenous protein-coding sequence, such that the growth enhancing agent is expressed as a fusion protein. The presence of all or part of the second exogenous protein can enhance or facilitate the targeting, stability, acceptance and/or processing of the growth enhancing agent within the cells of the mollusk.

Nucleic acid sequences are introduced into the germline of a mollusk embryo to generate a transgenic mollusk. A transgenic mollusk is one in which all of the somatic and germline cells (with the possible exception of a few cells subject to somatic mutation) contain at least one copy of an integrated transgene, the transgene having been introduced into the germline of the mollusk or an ancestor of the mollusk at an early embryonic stage. For example, the nucleic acid can be introduced into individual mollusk embryos at the one-cell (i.e., zygote) or two-cell stage. Introducing DNA at the one-cell stage results in a higher probability of germline incorporation. Incorporation at later stages often results in a mosaic mollusk, only some of whose cells have integrated the transgene. Subsequent breeding is then required to isolate true transgenic mollusks.

Methods of introducing exogenous nucleic acid sequences into embryonic cells include microinjection (*see* U.S. Patent No. 4,873,292), electroporation, viral transduction and the like. For example, nucleic acids encoding growth enhancing agents can be introduced into mollusks using the same procedure as described for introducing such nucleic acids into transgenic fish. See, e.g., Dunham, et al., supra, Chen, et al., supra. Alternatively, the exogenous nucleic acid segment is introduced into the mollusk by electroporation of a fertilized mollusk egg. The conditions for electroporation are the same as those conventionally used for electroporation of nucleic acids into mammalian cells. See Sambrook et al., supra. Occasionally, incorporation of an exogenous DNA disrupts an important cellular function. However, such

mollusks are easily eliminated, either by premature death, deformity or other distinguishable features such as reduced size and weight.

The presence of an integrated transgene in the genome of a mollusk is confirmed by extracting DNA from a tissue biopsy and analyzing the DNA for the presence of the nucleic acid sequence, e.g., by Southern analysis of DNA using probes specific for the exogenous nucleic acid sequence of the growth enhancing agent. Expression of the transgene is confirmed by Northern analysis of RNA from the biopsy using a similar probe. Alternatively, a protein extract of the biopsy is analyzed by Western blotting, radioimmunoassay or ELISA using an antibody to the growth enhancing factor being expressed as the probe. True transgenic status is established by transmission of the transgene from P1 mollusks to F1 progeny. The P1 mollusks can be bred either with other P1 mollusks or with nontransgenic mollusks. Interbreeding of transgenic mollusks can be used to establish a mollusk line that is homozygous for the integrated transgene.

III. Methods of Culturing Pearls

The invention provides methods for culturing pearls by enhancing the growth of the mollusks involved in the pearl culturing process. In modern commercial pearl culturing, a foreign body, or "nucleus" is introduced or implanted into the gonadal tissue of a mollusk. The mollusk receiving this nucleus is termed the mother or acceptor mollusk. Nuclei include any foreign body around which the mollusk produces a nacre. Such nuclei are generally small rounded objects, such as fragments of shells from other mollusks, metallic spheres, or other similarly shaped objects. The size and shape of such nuclei depends upon the size and shape of the pearl desired. Thus the invention provides custom shaped nuclei for producing pearls of any desired shape.

Generally, a piece of tissue from the mantle portion of a mollusk is also implanted in the gonadal tissue of the acceptor mollusk along with the nucleus. The mollusk from which this mantle tissue is derived, is termed the "donor"

mollusk. Although generally the mantle tissue is taken from a separate donor mollusk, the mantle tissue can be derived from the acceptor mollusk and reimplanted along with the nucleus in the gonadal tissue of that acceptor mollusk. Thus, in some instances, the acceptor mollusk can also be the donor mollusk. Similarly, although preferably the acceptor mollusk and the donor mollusk are the same species, this need not be the case. The implantation of the nucleus and donor mantle tissue is carried out by conventional methods routine in pearl culturing processes.

Optionally, a nucleus can be implanted between the shell and mantle of the acceptor mollusk, e.g., in the production of mabe pearls. Where this is the case, the implantation of the donor mantle tissue can be omitted.

A. Exposing Mollusks to Exogenous Growth Enhancing Agent

One embodiment of the present invention provides a method for culturing pearls comprising exposing pre-adult mollusks, e.g., juvenile, spat or larval mollusks, to an effective amount of a growth enhancing agent, whereby the growth rate of the mollusk is enhanced. When the mollusk reaches adult size, a nucleus and optionally, mantle tissue from a donor mollusk is introduced into the mollusk. The mollusk is then cultured and pearls are harvested therefrom. Whether a mollusk has reached adult size is dependent upon industry standards for a particular pearl producing mollusk, and as a result, varies from species to species. For example, the larger species of *Haliotis* have a larger adult size than the more typical *Pinctada maxima* oyster species.

The raising of mollusk spat for use in pearl culturing processes, herein termed "preculturing," can take upwards of 24 months. The present invention expedites the preculturing aspect of pearl culturing by enhancing the growth rate of juvenile or larval mollusks. As described for other aspects of the instant invention, the exposing of mollusk spat to an effective amount of growth enhancing agent can comprise immersing the spat in a solution comprising growth enhancing

agent at a concentration of from about 1 X 10^{-9} M (1nM) to about 1 X 10^{-2} M (10mM). More preferred are solutions wherein the growth enhancing agent is present in a concentration of from about 1 X 10^{-6} M (1μM) to about 1 X 10^{-4} M (100μM). Further, such immersion treatments should preferably comprise immersing the spat in the composition comprising the growth enhancing agent for from about 30 minutes to about 10 hours, and can be carried out as frequently as necessary to achieve the desired results. Preferably, such treatments are carried out daily, semi-weekly, weekly, biweekly or monthly during a portion of, or throughout the preculturing process.

In another embodiment, the present invention provides a method of culturing pearls comprising the steps of introducing into an acceptor mollusk, a nucleus and, optionally, mantle tissue from a donor mollusk, and exposing the acceptor mollusk to an effective amount of a growth enhancing agent. The acceptor mollusk is then cultured, and pearls are harvested therefrom. Again, where the nucleus is implanted between the oyster shell and the mantle of the acceptor mollusk, the implantation of mantle tissue from the donor mollusk can be omitted.

The exposing of the acceptor mollusk to the growth enhancing agent may take a variety of forms. In one embodiment, the implanted acceptor mollusk to be exposed to the growth enhancing agent is immersed in a solution which comprises an effective amount of the growth enhancing agent. This immersion treatment is carried out in substantially the same fashion as the treatment described for juvenile or larval oysters. Again, these treatments are preferably carried out daily, semi-weekly, weekly, biweekly or monthly during a portion of, or throughout the pearl culturing process. More preferably, the treatments are carried out during the natural growing season of the mollusk.

In an alternative embodiment, the solution comprising an effective amount of the growth enhancing agent described above is manually introduced between the valves of the implanted acceptor mollusk. In one embodiment, the solution comprising the growth enhancing agent as described above is directly

injected into the body or pallial space of the mollusk. While these injections are generally carried out on an implanted acceptor mollusk, these injections may be performed prior to implantation. These injections can be carried out by a number of methods, i.e., inserting a syringe needle between the opposing valves of the acceptor mollusk (a natural gap exists between valves in some oyster species, i.e., *Pinctada maxima*), filing a notch to produce a small hole in the junction of the two valves, or drilling a small hole in a valve so as to allow the injection of growth enhancing agent between the valves. Preferably, the solution comprising the growth enhancing agent is injected in a volume of from about 0.5 to about 2.0 mL. More preferably, the injection is into the pallial or extrapallial spaces of the mollusk so as not to penetrate the organs or tissue which comprise the body of the mollusk. Again, the injection treatments are carried out daily, semi-weekly, weekly, biweekly or monthly.

Effective amounts of growth enhancing agents in the above described solutions vary with the particular growth enhancing agent used. In general, effective amount is defined as a combination of concentration of growth enhancing agent in the solution to which the mollusk is exposed, the length of the individual exposure treatments and number and frequency of exposure treatments.

The growth enhancing agents are usually present in the above described solutions at a concentration of about 1×10^{-9} M (1nM) to about 1×10^{-2} M (10mM). More preferred are solutions wherein the growth enhancing agent is present in a concentration of from about 1×10^{-6} M (1 μM) to about 1×10^{-4} M (100 μM). Preferably, the growth enhancing agent is dispersed in seawater or other appropriate saline solutions.

In a related embodiment, the present invention also provides for the insertion of a controlled release composition comprising a growth enhancing agent in the tissue of the implanted acceptor mollusk. Controlled release compositions are generally in the form of a tablet, capsule or liposome such that the composition is retained within the tissue of the mollusk while releasing a constant low level of the growth

enhancing agent. These controlled release compositions generally incorporate the growth enhancing agent in a matrix such that the composition can release effective amounts of the growth enhancing agent over a sustained period of time. Specifically, the growth enhancing agents are contained within beads, capsules, or microcapsules, surrounded by a barrier which degrades slowly over time to sustain a constant, low level release of the growth enhancing agent into the surrounding environment, without producing degradation products harmful to the mollusk. Compositions useful as barriers are generally commercially available and include, for example, those compositions which are generally used in pharmaceutical applications. *See* Heller et al., *Advanced Drug Delivery Rev.* 10:163-204 (1993) and Tyrrell et al., *Biochim. Biophys. Acta* 457:259-302 (1976). Alternatively, the growth enhancing agents can also be adsorbed to a matrix whereby the growth enhancing agent is slowly released from the matrix in the culturing environment.

Preferably, the controlled release compositions are inserted into the body or tissue of the acceptor mollusk. Preferably, the controlled release composition can be inserted directly into the gonadal tissue of the mollusk so as to create a region of higher concentration of the growth enhancing agent in the immediate area of pearl formation.

In addition to allowing exposure of the acceptor mollusk to the growth enhancing agent with minimal manual manipulation of the mollusk, the slow dissolving nature of these controlled release compositions prevents them from acting as a nucleus for nacre formation.

Following culturing, pearls are harvested from the acceptor mollusks by conventional methods. These mollusks can then be reimplanted for repeated pearl culturing, if desired.

B. <u>Pearl Culturing with Transgenic Mollusks Capable of Expressing Growth Enhancing Agents</u>

As described previously, the modern pearl culturing process generally comprises the implantation or insertion of a nucleus and a piece of mantle tissue into the gonadal tissue

of the acceptor mollusk. However, this process may vary, for example, where mabe pearls are desired.

In a particular embodiment of the present invention, the nucleus and mantle tissue from a donor mollusk are implanted into a transgenic acceptor mollusk. The transgenic acceptor mollusk comprises an exogenous nucleic acid sequence which comprises a segment encoding a growth enhancing agent operably linked to a promoter sequence. The transgenic acceptor mollusk is thereby capable of expressing the growth enhancing agent.

Alternatively, the mantle tissue which is implanted into the acceptor mollusk is obtained from a transgenic donor mollusk. The donor mollusk then comprises the exogenous nucleic acid sequence comprising a segment encoding a growth enhancing agent operably linked to a promoter sequence. The implanted cells from the donor mollusk are thus capable of expressing the growth enhancing agent. This particular embodiment is particularly useful where it is desirable to avoid releasing a transgenic mollusk species into the environment. Therefore, this embodiment is readily be practiced on the scale of a commercial pearl culturing operation.

Although the above embodiments are stated in terms of either a transgenic acceptor mollusk or a transgenic donor mollusk, either or both the acceptor and donor mollusks can be transgenic, i.e., capable of expressing an exogenous growth enhancing agent.

The present invention is further illustrated by the following examples. These examples are merely to illustrate aspects of the present invention and are not intended as limitations of this invention.

EXAMPLES

All reagents used herein are available commercially unless otherwise noted.

<u>Example 1: General Oyster Production</u>

For a number of the following examples, oysters at various stages of development are used. Accordingly, the initial example describes the raising and culturing of oysters from egg cells to adult oysters.

Typical broodstock oysters are collected from the wild just prior to the natural spawning season. These oysters are kept in flow through or recirculating seawater at temperatures just below the natural spawning temperature. These are typically from about 25 to about 30°C. When judged to be in spawning condition by visual inspection of the gonads, the temperature is increased by from about 5°C to about 8°C. This temperature increase usually induces spawning.

Upon induction of spawning, oysters are identified as male or female and placed in separate vessels accordingly. When spawning is complete, the oysters are removed from their respective tanks, and the sperm and egg suspensions are collected and mixed. Once fertilized, the eggs are transferred to another tank to begin the larval culture.

Fertilized eggs are stocked at a density of from about 20 to about 30 eggs per ml and kept in aerated tanks for 24 to 48 hours, during which time the eggs hatch. The larvae from the eggs are collected on sieves and placed in tanks at a density of about 20 larvae per ml, and fed algae. The feed is typically a mixture of diatoms and *Isochrysis galbana*, at a density of 20,000 to 30,000 cells/ml in the larval tanks.

As the larvae grow, their densities are reduced. Typically, when the larvae reach pediveliger stage, their density is reduced to 2 to 3 per ml. For metamorphosis, some species require addition of a substrate upon which to attach during the process, while others simply settle to the bottom of the tank. Metamorphosis may be chemically induced in some species.

Following metamorphosis, the young oysters, now termed spat, are moved into flow through containers, e.g., upwellers or downwellers, and fed by coarsely filtered seawater until reaching adulthood.

Example 2: Immersion

A. Enhancing Spat Growth

In order to assess the effect of growth promoters on juvenile oysters, the following experiment was performed:

Juvenile pearl oysters (*Pinctada margeritifera*) approximately 1 mm in length, were obtained from a commercial source. These juveniles were maintained in closed recirculating aquaria at 28°C, and fed *Thallasiosira* and *Isochrysis*. A subset of the juveniles was immersed in seawater containing 10^{-6}M insulin while another group was immersed in seawater alone. After one week, the treated group showed 30% greater growth than the untreated group. Continued weekly immersions resulted in continued accelerated growth of the juveniles over that of the untreated group. No mortality was observed in either group.

B. Enhancing Acceptor Shell Growth and Pearl Production

Adult oysters of approximately the same age are obtained from commercial hatcheries. A spherical nucleus having a diameter of 10 mm, and a 25 mm^2 sample of mantle tissue from another adult oyster is implanted into these oysters. Half of the oysters given these implants are treated by immersion in a ten fold volume of filtered ambient seawater containing from about 10 μM porcine insulin. The treatment is carried out every 14 days throughout the growing season and ceased in nongrowing periods. The remaining half is the control group, subject to the same manipulations but absent any insulin contact.

After treatment for 12 months, the oysters in the treated group show a greater growth percentage than the oysters in the nontreated group. The oysters of both groups are opened and the pearls produced therein are harvested, dried, weighed and their diameter is measured. Pearls obtained from the oysters subject to the treatment with insulin show greater dry weight and larger diameter than those produced by the control group.

Example 3: Controlled Release Composition

A slow-release insulin capsule such as described by Heller, *supra* is inserted into the tissue or gonad of a test group of oysters. Concurrently with the insertion of the insulin capsule, a nucleus of 10 mm in diameter and a 25 mm^2 sample of mantle tissue from a donor oyster are inserted into a separate incision in the gonadal tissue of the test oyster. The oysters are closed and returned to normal culture conditions. After culturing for 12 months, the test oysters are opened and their pearls are harvested. Pearls harvested from these test oysters have a larger diameter and a greater dry weight than pearls produced by the control group.

Example 4: Injection of the Growth Enhancing Agent

Juvenile *Pinctada maxima* oysters are removed from their culture conditions. An 18-gauge needle is inserted through the dorsal notch into the pallial space of each oyster. One ml of 100μM insulin solution is injected into the oyster. The injected oysters are held out of the water for one hour. After treatment, the oysters are returned to their normal culture conditions. The injections are repeated every 14 days during the growing season. After 12 months, the oysters show greater percent growth than the oysters of the control group.

Example 5: Generating Transgenic Oysters

The cDNA sequence of human insulin has been described. See, Gen Bank Acc. No. L15440. The cDNA coding sequence including 3' and 5' untranslated regions and a polyadenylation signal is modified by the addition of SalI linkers and ligated to a Rous Sarcoma Virus long terminal repeat (RSV-LTR) promoter/enhancer sequence at the SalI site in a pRSV-2 vector, as described by Gorman et al, Proc. Natl Acad. Sci. (USA) 79:6777-6781 (1982). The cDNA coding sequence is thereby linked to 580 bp of the LTR sequence and 3.7 kb of pRSV flanking sequence. After propagation of the vector, the vector is linearized to provide a transgene suitable for injection.

The linearized transgene is electroporated into fertilized eggs from *Pinctada maxima* obtained from a commercial hatchery or by laboratory spawning and fertilization. The eggs are cultured to spat and juvenile oysters. The presence of the RSV-LTR-Insulin transgene is assayed from a biopsy of the oyster tissue. Chromosomal DNA is extracted and amplified using primers hybridizable to the transgene. The amplification product is detected by gel electrophoresis. Expression of human insulin is detected by Western blotting of a protein extract from the biopsy. Founder transgenic oysters are interbred to generate transgenic progeny, whose transgenic status and expression is assayed using the same methods as described for the founder oysters.

Juvenile transgenic oysters which express the human insulin sequence are cultured under the same conditions as a control group of normal oysters of approximately equal size and age. After culturing for 12 months, the transgenic oysters show a higher percent growth than the oysters of the control group.

Example 6: Pearl Culturing Using Transgenic Oysters

To show the effects of the expression of growth enhancing agents on the pearl culturing process, two subsets of oysters are provided. Set 1 comprises the oysters of Example 5 which demonstrate enhanced growth rates relative to the control group. Set 2 comprises "normal" *Pinctada maxima* oysters, i.e., nontransgenic oysters.

The oysters are implanted with a spherical nucleus 10 mm in diameter, and a 25 mm^2 sample of donor mantle tissue. The following sets of acceptor oysters results:

1. Transgenic acceptor oyster, normal donor oyster.
2. Normal acceptor oyster, transgenic donor oyster.
3. Transgenic acceptor oyster, transgenic donor oyster.
4. Normal acceptor oyster, normal donor oyster (control).

The acceptor oysters are cultured in a marine environment for 12 months. At the completion of the culturing process,

the pearls obtained from the oysters of groups 1, 2 and 3 are larger in diameter and have a greater dry weight than those produced by the control group.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted.

<u>WHAT IS CLAIMED IS</u>:

1. A method of enhancing the growth of a pearl producing mollusk, said method comprising introducing into the germline of said mollusk a nucleic acid sequence, said nucleic acid sequence comprising a segment which encodes a growth enhancing agent, said segment being operably linked to a promoter sequence whereby said segment is capable of expressing said growth enhancing agent in said mollusk.

2. The method of claim 1, wherein said mollusk is selected from the group consisting of *Pteria penguin, Pinctada maxima, Pinctada margeritifera, Pinctada martensi fucata, Pinctada radiata, Pinctada vulgaris, Pinctada fucata, Pinctada maculata, Pinctada albina, Unionides sp.* and *Haliotis sp.*

3. The method of claim 1, wherein said growth enhancing agent is selected from the group consisting of insulin, molluscan insulin-like peptide and growth hormone.

4. The method of claim 1, wherein said promoter sequence is an RSV-LTR or mouse metallothionein-I promoter sequence.

5. A transgenic mollusk, said mollusk having a germline comprising an exogenous nucleic acid sequence, said exogenous nucleic acid sequence comprising a segment which encodes a growth enhancing agent, said segment being operably linked to a promoter sequence, and wherein said segment is capable of expressing said growth enhancing agent in said mollusk.

6. The transgenic mollusk of claim 5, wherein said promoter sequence is an RSV-LTR or mouse metallothionein-I promoter sequence.

7. The transgenic mollusk of claim 5, wherein said growth enhancing agent is selected from the group consisting of insulin, molluscan insulin-like peptide and growth hormone.

8. The transgenic mollusk of claim 5, wherein said mollusk is selected from the group consisting of *Pteria penguin, Pinctada maxima, Pinctada margeritifera, Pinctada martensi fucata, Pinctada radiata, Pinctada vulgaris, Pinctada fucata, Pinctada maculata, Pinctada albina, Unionides sp.* and *Haliotis sp.*

9. A method of culturing pearls comprising:
(a) introducing a nucleus into an acceptor mollusk;
(b) exposing said acceptor mollusk to an effective amount of a growth enhancing agent;
(c) culturing said acceptor mollusk; and
(d) harvesting pearls from said acceptor mollusk.

10. The method of claim 9, further comprising introducing into said acceptor mollusk, mantle tissue from a donor mollusk.

11. The method of claim 10, wherein said acceptor mollusk and said donor mollusk are selected independently from the group consisting of *Pinctada maxima, Pinctada margeritifera, Pinctada martensi fucata, Pinctada radiata, Pinctada vulgaris, Pinctada fucata, Pinctada maculata, Pinctada albina, Unionides sp.* and *Haliotis sp.*

12. The method of claim 9, wherein said acceptor mollusk is exposed to said growth enhancing agent by immersing said acceptor mollusk in a solution comprising said growth enhancing agent at a concentration of from about 1 nM to about 10 mM of the growth enhancing agent.

13. The method of claim 9, wherein said acceptor mollusk is exposed to said growth enhancing agent by immersing said mollusk in a solution comprising said growth enhancing agent at a concentration of from about 0.1 μM to about 100 μM of the growth enhancing agent.

14. The method of claim 9, wherein said acceptor mollusk is exposed to said growth enhancing agent by introducing a composition comprising said growth enhancing agent into the interior of said acceptor mollusk.

15. The method of claim 14, wherein said introducing of said composition comprises injecting said composition into the interior of said acceptor mollusk.

16. The method of claim 14, wherein said composition comprising said growth enhancing agent is a controlled release composition.

17. The method of claim 9, wherein said growth enhancing agent is selected from the group consisting of insulin, molluscan insulin-like peptide, and growth hormone.

18. A method of culturing pearls comprising:

(a) introducing into an acceptor mollusk, a nucleus and mantle tissue from a donor mollusk, said donor mollusk having a germline comprising a nucleic acid sequence which encodes a growth enhancing agent, said sequence being operably linked to a promoter sequence whereby said segment is capable of expressing said growth enhancing agent in said mollusk;

(b) culturing said acceptor mollusk;

(c) harvesting pearls from said acceptor mollusk.

19. The method of claim 18, wherein said acceptor mollusk and said donor mollusk are selected independently from the group consisting of *Pteria penguin, Pinctada maxima, Pinctada margeritifera, Pinctada martensi fucata, Pinctada radiata, Pinctada vulgaris, Pinctada fucata; Pinctada maculata, Pinctada albina*, *Unionides sp*. and *Haliotis sp*.

20. The method of claim 18, wherein said growth enhancing agent is selected from the group consisting of insulin, molluscan insulin-like peptide and growth hormone.

21. The method of claim 19, wherein said promoter sequence is an RSV-LTR or a mouse metallothionein-I promoter sequence.

22. A method of culturing pearls comprising:

(a) introducing a nucleus into an acceptor mollusk, said acceptor mollusk having a germline comprising a nucleic acid sequence, said nucleic acid sequence comprising a segment which encodes a growth enhancing agent, said segment being operably linked to a promoter sequence whereby said segment is capable of expressing said growth enhancing agent in said acceptor mollusk;

(b) culturing said acceptor mollusk; and

(c) harvesting pearls from said acceptor mollusk.

23. The method of claim 22, further comprising introducing into said acceptor mollusk, mantle tissue from a donor mollusk.

24. The method of claim 22, wherein said growth enhancing agent is selected from the group consisting of insulin, insulin-like growth factor and growth hormone.

25. The method of claim 22, wherein said promoter sequence is an RSV-LTR or a mouse metallothionein-I promoter sequence.

26. The method of claim 23, wherein said acceptor mollusk and said donor mollusk are selected, independently, from the group consisting of *Pteria penguin*, *Pinctada maxima*, *Pinctada margeritifera*, *Pinctada martensi fucata*, *Pinctada radiata*, *Pinctada vulgaris*, *Pinctada fucata*, *Pinctada maculata*, *Pinctada albina*, *Unionides sp.* and *Haliotis sp.*

27. A method of culturing pearls comprising:

(a) exposing a mollusk to an effective amount of a growth enhancing agent, whereby the growth rate of said mollusk is enhanced;

(b) introducing a nucleus into said mollusk;
(c) culturing said mollusk;
(d) harvesting pearls from said mollusk.

28. The method of claim 27, further comprising introducing into said mollusk, mantle tissue from a donor mollusk.

29. The method of claim 28, wherein said mollusk and said donor mollusk are selected independently from the group consisting of *Pteria penguin, Pinctada maxima, Pinctada margeritifera, Pinctada martensi fucata, Pinctada radiata, Pinctada vulgaris, Pinctada fucata, Pinctada maculata, Pinctada albina, Unionides sp.*, and *Haliotis sp.*

30. The method of claim 27, wherein said mollusk is exposed to said growth enhancing agent by immersing said mollusk in a solution comprising said growth enhancing agent at a concentration of from about 1 nM to about 10 mM of the growth enhancing agent.

31. The method of claim 27, wherein said growth enhancing agent is selected from the group consisting of insulin, molluscan insulin-like peptide and growth hormone.

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01K 61/00; C12N 5/00, 15/00
US CL :119/234, 236, 244; 435/172.3; 800/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 119/234, 236, 244; 435/172.3; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, EMBASE, MEDLINE, DERWENT, AGRICOLA, ASFA, CAS, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TABURIAUX, "PEARLS Their Origin, Treatment and Identification" published 1985 by Chilton Book Company (Radnor, PA), pages141-160.	9-31
Y	Aquaculture, Volume 39, issued 1984, Morse, "BIOCHEMICAL AND GENETIC ENGINEERING FOR IMPROVED PRODUCTION OF ABALONES AND OTHER VALUABLE MOLLUSCS", pages 263-282, see the entire document.	1-31
Y	Biology Bulletin, Volume 181, issued December 1991, Paynter et al, "Biological Activity of Biosynthetic Rainbow Trout Growth Hormone in the Eastern Oyster, Crassostrea virginica", pages 459-462, see the entire document.	1-31

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

22 FEBRUARY 1996

Date of mailing of the international search report

13 MAR 1996

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Transactions of the American Fisheries Society, Volume 116, issued 1987, Dunham et al, "Transfer of the Metallothionein-Human Growth Hormone Fusion Gene into Channel Catfish", pages 87-91, see the entire document.	1-31
Y	Aquatic Science and Fisheries Abstracts, August 1994, Powers et al, "Genetic engineering a fast growing strain of the red abalone Haliotis rufescens", abstract number 124-21838, 3rd International Marine Biotechnology Conference: Program Abstracts and List of Participants, page 70, see the entire abstract.	1-31